REMARKS/ARGUMENTS

Claims 1-5, 7-13 and 15-30 are pending in this application. Claims 6 and 14 were previously cancelled and claims 8-12, 20 and 21 were previously withdrawn. Claims 1-5, 7, 13, 15-19, and 22-30 stand rejected.

I. Rejection Under 35 U.S.C. § 112, first paragraph – Enablement Rejection.

Claims 1-5, 7, 13, 15-19, 22-24 and 26-30 stand rejected under 35 U.S.C. §112, first paragraph as allegedly containing subject matter which was not described in the specification in such way as to enable one skilled in the art to which it pertains, or with which it is most nearly connected, to make and/or use the invention. As described in detail below, no *prima facie* case of lack of enablement has been established. Furthermore, even if a *prima facie* case of lack of enablement has been established, which is denied, Applicants previously rebutted it.

It is settled law that a specification disclosure which contains a teaching of the manner and process of making and using the invention in terms which correspond in scope to those used in describing and defining the subject matter sought to be patented must be taken as in compliance with the enablement requirement of the first paragraph of § 112 unless there is reason to doubt the objective truth of the statements contained therein. MPEP 2164.04 citing *In re Marzocchi*, 58 C.C.P.A. 1069, 439 F.2d 220, 223, 169 U.S.P.Q. (BNA) 367, 369 (CCPA 1971). Thus, the PTO has the initial burden of challenging a presumptively correct assertion of enablement in the disclosure. Only after the PTO provides evidence showing that one of ordinary skill in the art would reasonably doubt the asserted utility does the burden shift to the applicant to provide rebuttal evidence sufficient to convince such a person of the invention's asserted utility. *See In re Bundy*, 642 F.2d 430, 433, 209 U.S.P.Q. (BNA) 48, 51 (CCPA 1981).

The Examiner has alleged for various reasons that the instant claims are not enabled by the present disclosure. In particular, the Examiner purports that the state of the art would require undue experimentation for administering peptides to induce immune tolerance to prevent/delay the onset of Type 1 diabetes in humans because such methods were unpredictable at the time of the present invention. However, the PTO has not provided any credible evidence showing that

one of ordinary skill in the art would ***reasonably doubt*** the asserted utility of the claimed invention and has therefore not met its initial burden. The Office Action at page 2 states that:

> [w]hile the mechanism of action for the method of the instant claims is not disclosed, it appears to require inducing tolerance to GAD and altered GAD 'derived' peptides. Tolerance-inducing peptide immunotherapy is well known in the immunological arts. In some cases significant results have been demonstrated in in-bred small animal models. However said results have not been repeated in human trials.

As an initial matter, Applicants point out that tolerance induction as a mechanism of action is not a limitation of the present claims. Furthermore, as discussed in detail below, the sum total the evidence provided by the Patent Office shows that, using fundamentally different therapeutic agents than presently claimed, tested in diseases other than Type 1 diabetes as presently claimed, some researchers have achieved tolerance results in animal models that have been difficult to reproduce in humans. See, Office Action at page 3. At most, these references are only tangentially related to the presently claimed invention and simply do not cast any doubt, let alone any reasonable doubt, on the presently clamed invention which entails an altogether different therapeutic agent and altogether different disease state than those discussed in the references relied upon by the PTO. This is simply not enough to establish a *prima facie* case of lack of enablement.

Based on the logic used in the instant rejection, the use of a novel compound to treat a given cancer would be unpatentable in the absence of human data if prior treatment of an altogether different cancer with a different compound had shown success in animals but failed to achieve FDA approval. This is neither the law nor sound policy. Applicants respectfully submit that the burden of challenging the presumptively correct assertion of the manner of making and using the invention has not been met.

A. No *Prima Facie* Case of Lack of Enablement has been Established.

i. Post-filing Date References Cannot Be Used in an Enablement Rejection.

MPEP 2164.05(a) and *In re Hogan* (559 F.2d 595, 605, 194 USPQ 527, 537 (CCPA 1977)) make clear that post-filing date references should not be used to demonstrate that the patent is non-enabling. Two narrow exceptions to this rule exist in cases where (1) a later-dated

reference provides evidence of what one skilled in the art would have known **on or before** the effective filing date of the patent application or (2) if a later-dated reference **discloses the claimed invention**. *Id.* Specifically, the state of the art existing at the filing date of the application is used to determine whether a particular disclosure is enabling as of the filing date. *Chiron Corp. v. Genentech Inc.*, 363 F.3d 1247, 1254 70 USPQ2d 1321, 1325-26 (Fed. Cir. 2004). Despite decades of settled case law on this point, "[it] remains the Examiner's position that the use of post-filing references in this case is proper." Office Action at page 6.

In the Office Action, the Examiner attempts to overturn years of existing case law by setting forth that any post-filing date reference can be used in an enablement rejection. Specifically, the Examiner suggests that a post-filing date reference that provides evidence of what one skilled in the art learned after the filing date can be used in an enablement rejection. This is not the law. As was recognized by the Federal Circuit in *Plant Genetic Systems v. Dekalb Genetics Corporation*, 315 F.3d 1335 (Fed. Cir. 2003), the *Hogan* case stands for the proposition that one could not use a later-existing state of the art to invalidate a patent that was enabled for what it claimed at the time of filing outside of the exceptions mentioned above. Specifically, the *Hogan* court stated that "[t]his court has approved use of later publications as evidence of the state of art existing on the filing date of an application. That approval **does not extend**, however, to the use of a later ... publication disclosing a later (1962) existing state of the art in testing an earlier (1953) application for compliance with § 112, first paragraph. The difference may be described as that between the permissible application of later knowledge about art-related facts existing on the filing date and the impermissible application of later knowledge about later art-related facts ... which did not exist on the filing. *Hogan*, 559 F.2d at 605. As such, the post-filing date references and later state of the art relied on by the Examiner cannot be used to challenge enablement as of the time the instant application was filed.

Additionally, in an attempt to support the misplaced belief that any post-filing date reference may be used to reject claims for lack of enablement, the Examiner points to MPEP 2164.05(a) which provides in part:

> "If individuals of skill in the art state that a particular invention is not possible years after the filing date, that would be evidence that the disclosed invention was not possible at the time of filing and should be considered. In *In re Wright*, 999 F2d 1557, 1562, 27 USPQ2d 1510, 1513-14 (Fed Cir. 1993) an article published 5 years after the filing date of the application adequatley supported the examiner's

> position that the physiological activity of certain viruses was sufficeinty unpredictable so that a person skilled in the art would not have believed that the success with one virus and one animal could be extrapolated successfully to all viruses with all living organisms."

Applicants respectfully point out that the Examiner has attempted to apply *In re Wright* to support his position that ***any*** reference that post dates the effective filing date of the instant application may be used in a rejection of the claims for lack of enablement. Such an interpretation and application of *In re Wright* is clearly erroneous. Applicants point out that in *In re Wright* the patentee described a process for the production of a recombinant vaccine which conferred immunity against the RNA tumor virus Prague Avian Sarcoma Virus (PrASV) but sought to patent claims directed to processes for producing recombinant vaccines against ***any*** pathogenic avian RNA viruses. The Court in *In re Wright* held that such claims were not enabled in view of a reference dated 5 years after the filing date of Wright's application – the reference disclosing that recombinant vaccines to AIDS retroviruses (an RNA virus) did not produce antibodies that could prevent viral infectivity. Thus, the **very same invention that the patentee sought to patent** (*i.e.*, processes for producing recombinant vaccines against any pathogenic avian RNA viruses) **was disclosed in the later filed publication** as not working for certain RNA viruses. In other words, the patentee sought to patent a genus when in fact the later filed reference demonstrated that a particular species within the genus was not enabled. Accordingly, *In re Wright* held that a reference published after the effective filing date of a patent application may be used in a rejection of the claims for lack of enablement **only** where the later filed reference disclosed the claimed invention. In contrast, the invention embodied by the instant claims is not disclosed by any of the references cited by the Examiner. The instant invention is directed to methods for preventing or delaying onset of Type-1 diabetes in a subject by, *inter alia*, administering to the subject a pharmaceutically acceptable composition comprising a soluble fusion protein comprising at least one immunoglobulin having at least one variable region and at least one peptide inserted within the at least one variable region, wherein the at least one peptide is GAD2 represented by SEQ ID NO: 4. By contrast, the references relied upon by the Examiner are only tangentially related to the presently claimed invention which entails an altogether different therapeutic agent and altogether different disease state than those discussed in the references relied upon by the PTO. Accordingly, the post-filing date

references cited by the Examiner cannot be used to reject to the instant claims for lack of enablement because the claimed invention is not disclosed in any of the references cited by the PTO.

The references being relied on for lack of enablement are as follows (publication year in parentheses):

1) Legge (1998);

2) Marketletter (1999);

3) Dong (1999);

4) Pozzilli (2000);

5) Goodnow (2001);

6) WO 02/053092 (July 2002);

7) Skyler (2005);

8) Kraus and Mayer (2005);

9) Leslie (2010);

10) Bell (2008);

11) von Herrath and Nepom (2009); and

12) Van der Worp (2010).

Each of references 6-12 were published **after** the priority date of the present application and are impermissibly being relied on for what one skilled in the art would have known **on or before** the effective filing date of the patent application. Moreover, none of references 6-12 discloses the claimed invention and thus do not fall within the narrow exception set forth in Hogan. As such, these references should not be used in an enablement rejection.

Moreover, the Examiner asserts that the references (1-12 above) cited to support the rejection of the claims for lack of enablement cannot be attacked individually but rather must be considered in combination. In particular, the Examiner states that "[i]n response to Applicant's argument against the references individually, one cannot show ***nonobviousness*** by attacking the references individually where the rejections are based on combinations of references. See *In re*

Keller, 642 F.2d 413, 208 USPQ 871 (CCPA 1981); *In re Merck & Co.*, 800 F.2d 1091, 231 USPQ 375 (Fed. Cir. 1986)." Office Action at page 6. Applicants point out that the Examiner is attempting to apply case law concerning the combination of references in a rejection under 35 U.S.C. 103 and to a rejection for lack of enablement. Applicants submit that nothing in *In re Keller*, *In re Merck & Co.* or the MPEP support the Examiner's proposition that references may be considered in combination to support a rejection for lack of enablement. Moreover, the Examiner states that "the ten references combined render the claimed invention non-enabled." Office Action at page 6. Thus, by having to rely on a combination of references to reject the claims for lack of enablement, the Examiner admits that each reference taken singly does not call into question the enablement of the instant claims. Accordingly, for at least these reasons, the rejection of the instant claims for lack of enablement should be withdrawn.

For at least the foregoing reasons, no *prima facie* case of lack of enablement has been established. Reversal of this rejection is therefore respectfully requested.

ii. Pre-filing date references do not establish a *prima facie* case of lack of enablement.

As has been extensively discussed in the record, none of the pre-filing date references establish a *prima facie* case of lack of enablement. It is settled law that a specification disclosure which contains a teaching of the manner and process of making and using the invention in terms which correspond in scope to those used in describing and defining the subject matter sought to be patented must be taken as in compliance with the enablement requirement of the first paragraph of § 112 unless there is reason to doubt the objective truth of the statements contained therein. MPEP 2164.04 citing *In re Marzocchi*, 58 C.C.P.A. 1069, 439 F.2d 220, 223, 169 U.S.P.Q. (BNA) 367, 369 (CCPA 1971). Thus, the PTO has the initial burden of challenging a presumptively correct assertion of enablement in the disclosure. Only after the PTO provides evidence showing that one of ordinary skill in the art would reasonably doubt the asserted utility does the burden shift to the applicant to provide rebuttal evidence sufficient to convince such a person of the invention's asserted utility. *See In re Bundy*, 642 F.2d 430, 433, 209 U.S.P.Q. (BNA) 48, 51 (CCPA 1981).

The PTO has not provided any credible evidence showing that one of ordinary skill in the art would ***reasonably doubt*** the asserted utility of the claimed invention and has therefore not met its initial burden. Each of the pre-filing date references are addressed below:

- *Marketletter*; and *Pozzilli*

The Examiner alleges that practice of the instant claims would require undue experimentation because attempts to induce immune tolerance in humans have been unsuccessful as supported by *Marketletter* and *Pozzilli* which allegedly disclose failed experiments involving the use of peptides to induce immune tolerance. However, Applicants reiterate that *Marketletter* and *Pozzilli* simply do not disclose the administration of a fusion protein construct, let alone a fusion protein construct comprising GAD2 to as presently claimed. Specifically, *Marketletter* is a review of free peptides in multiple sclerosis and rheumatoid arthritis and *Pozzilli* evaluated insulin administration in diabetes. These references provide no evidence that a person of ordinary skill in the art at the time of filing of the instant application would have reasonably doubted the asserted utility of the presently claimed invention.

- *Dong*; *Legge*; and *Goodnow*

Additionally, the Examiner alleges that practice of the instant claims would require undue experimentation because *Dong*, *Legge* and *Goodnow* disclose that immune tolerance is not predictable. Again, none of *Dong*, *Legge* or *Goodnow* contain anything to call in to question the use of a fusion protein comprising GAD2 (in the presently claimed construct) for preventing or delaying type 1 diabetes in a subject as claimed. *Dong* is merely a general review of tissue graft transplant tolerance (unrelated to treatment of any autoimmune disorder let alone type 1 diabetes) and also contains nothing to call in to question use of a fusion protein construct as claimed for preventing or delaying type 1 diabetes in a subject that has undergone insulin autoantibody seroconversion. Additionally, *Legge* discloses the use of Ig constructs comprising PLP-LR (a protein implicated in MS). Moreover, *Goodnow* is a general review article about pathways for self-tolerance in auto-immune disorders. Again, none of these reference contain anything to call in to question the use of a fusion protein comprising the GAD2 peptide (in the presently claimed construct) for preventing or delaying the onset of type 1 diabetes.

At most, the references properly relied on by the Examiner are only tangentially related to the presently claimed invention and/or simply do not cast any doubt, let alone any reasonable

doubt, on the presently clamed invention. This is simply not enough to establish a *prima facie* case of lack of enablement. Withdrawal of the rejection is respectfully requested.

B. The Application Clearly Teaches How to Make and Use the Claimed Invention as is Required for Enablement.

Again, the enablement requirement consists of two prongs: first, the application must describe how to make the invention; second, the application must describe how to use the invention. 35 U.S.C., paragraph 1. The instant application clearly meets both prongs.

The instant claimed method is directed to preventing or delaying onset of Type 1 diabetes in a subject by, *inter alia*, administering to the subject a pharmaceutically acceptable composition comprising a soluble fusion protein comprising at least one immunoglobulin having at least one variable region and at least one peptide inserted within the at least one variable region, wherein the at least one peptide is GAD2 represented by SEQ ID NO: 4. The application contains everything need for the person of ordinary skill in the art to make and use the claimed invention.

Specifically, guidance as to how to make the claimed constructs is provided in the specification at pages 45, line 13 - page 47, line 3. General dosing guidance is provided in the specification at page 34, line 21- page 37, line 17. Guidance for determining whether administration of a claimed fusion protein effectively prevented or delayed diabetes in humans or mice is provided in the specification at page 42, line 3 - page 42, line 7 and page 45, lines 5-11. Guidance for determining if a subject has undergone insulin autoantibody seroconversion is provided in the specification at page 55, line 15 – page 56, line 24.

C. Even assuming, *arguendo*, that a *prima facie* case exists, Applicants previously rebutted it.

To further demonstrate that Applicants' claimed invention was enabled at the time of filing, Applicants previously submitted a declaration under 37 CFR 1.312 showing that the claimed method effectively prevents and/or delays the onset of Type 1 diabetes in the gold standard NOD mouse model for that disease. ("Zaghouani Declaration I"). As has been clearly established in the record, the NOD mouse model used in the experiment described in the Zaghouani Declaration I is considered the gold standard animal model for Type 1 diabetes, regardless of whether some unrelated agent in the past has shown efficacy in that model that did

not translate to humans. The pharmaceutical industry is replete with molecules that showed efficacy in gold standard models that failed to achieve FDA approval—this does not render later, different candidate molecules unpatentable for lack of enablement.

It was clear error to reject the instant claims under 35 U.S.C. § 112, first paragraph on the alleged basis that successful results in the gold standard animal model for Type 1 diabetes do not necessarily translate to humans or other species. Applicants have taught the public that the claimed soluble IgGAD2 construct can prevent or delay onset of Type 1 diabetes in a standard experimental animal and have thus made a significant and useful contribution to the art, even though it could eventually be determined that the compound is without value in the treatment of humans. These data demonstrate that the claimed invention was enabled when filed. MPEP 2164.05 and *In re Brana*, 51 F.3d 1560 (Fed. Cir. 1995).

i. Treatment of humans is not required.

The Examiner further takes the position that while tolerance-inducing peptide immunotherapy is well known in the immunological arts and has shown significant results in in-bred small animal models – the results have not been repeated in human trials. Office Action at page 2. The Examiner attempts to support this conclusion by citing to instances in which treatment (albeit again with fundamentally different types of therapeutic agents and in different diseases than Type 1 diabetes as discussed above) succeeded in animals but failed in humans—for example *Marketletter*. That position—also rejected by the Federal Circuit in *Brana*—is clear legal error.

In *Brana*, the PTO argued in the context of a 112, first paragraph rejection that *in vivo* test results in animals are not reasonably predictive of the success of the claimed compounds for treating cancer in humans. *Id* at 20. In response, the Federal Circuit stated:

> The Commissioner, as did the Board, confuses the requirements under the law for obtaining a patent with the requirements for obtaining government approval to market a particular drug for human consumption... proof of an alleged pharmaceutical property for a compound by statistically significant tests with standard experimental animals is sufficient to establish utility. *In re Krimmel*, 48 C.C.P.A. 1116, 292 F.2d 948, 953...In concluding that similar *in vivo* tests were adequate proof of utility the court in *In re Krimmel* stated: We hold as we do because it is our firm conviction that one who has taught the public that a compound exhibits some desirable pharmaceutical property **in a standard experimental animal has made a significant and useful contribution to the**

art, even though it may eventually appear that the compound is without value in the treatment of humans. *Id.* At 22. (emphasis added)

Again, the NOD mouse model used in the experiment described in the Zaghouani Declaration I is considered the gold standard animal model for Type 1 diabetes. It was clear error to reject the instant claims under 35 U.S.C. § 112, first paragraph on the alleged basis that successful results in the gold standard animal model for Type 1 diabetes do not necessarily translate to humans or other species. Applicants have taught the public that the claimed soluble IgGAD2 construct can prevent or delay onset of Type 1 diabetes in a standard experimental animal and have thus made a significant and useful contribution to the art, even though it could eventually be determined that the compound is without value in the treatment of humans.

For at least the foregoing reasons, no *prima facie* case of lack of enablement has been established. Even if a *prima facie* case of lack of enablement is deemed to have been established, which is not admitted, Applicants have rebutted it. Reversal of this rejection is therefore respectfully requested.

II. Obviousness Type Double Patenting Rejections.

Claims 1-5, 7, 13, 15-19, 22-24, and 26-30 stand provisionally rejected under the judicially created doctrine of obviousness-type double patenting over claims 1-7 and 13-16 of U.S. Serial N0. 11/290,070 and claims 1-7 and 13-16 of U.S. 11/425,084. In response, Applicants submit that they will address these provisional rejections upon resolution of the outstanding non-provisional rejections.

III. Rejection Under 35 U.S.C. § 112, first paragraph – Written Description Rejection.

Claims 1-5, 7, 13, 15-19, 22-24 and 26-30 stand rejected under 25 U.S.C. § 112, first paragraph as failing to comply with the written description requirement. In order to establish a *prima facie* case of lack of written description, the Examiner must show that the application as filed does not reasonably describe or convey to one of ordinary skill in the art, at the time of filing the application, that the inventor had possession of the claimed invention. MPEP § 2163.03. As stated by the Board, "the examiner has the initial burden of presenting evidence or

reasons why persons skilled in the art would not recognize in [the] specification disclosure a description of the invention defined by the claims. *Ex parte Sorenson*, 3 U.S.P.Q.2d 1462 (BPAI 1987). "It is not necessary that the application describe the claim limitations exactly,...but only so clearly that persons of ordinary skill in the art will recognize from the disclosure that appellants invented processes including those limitations." *In re Wertheim*, 541 F.2d 257 (CCPA 1976). Adequate description under the first paragraph of 35 U.S.C. § 112 does not require literal support for the claimed invention...Rather, it is sufficient if the originally-filed disclosure would have ***conveyed to one having ordinary skill in the art*** that an applicant had possession of the ***concept*** of what was claimed. *Ex parte Parks*, 30 U.S.P.Q.2d 1234 (BPAI 1994).

In the Office Action, the Examiner states that the specification and originally filed claims do not provide support for the invention as now claimed, specifically: (A) a method comprising administration of an immunoglobulin construct comprising a protein represented by SEQ ID NO: 4 (claims 1 and 13). As will be discussed in detail below, no *prima facie* case of lack of written description has been established and this rejection should be reversed.

Claims 1 and 13 each claim a method of preventing or delaying onset of Type 1 diabetes comprising, *inter alia*, administering to a subject a soluble fusion protein comprising at least one immunoglobulin having at least one protein fragment or peptide inserted in the variable region, wherein the protein fragment or peptide is (claim 1), or consists essentially of (claim 13), GAD2 represented by SEQ ID NO: 4.

Applicants point out that the specification as filed contains multiple descriptions of an immunoglobulin construct comprising GAD2/SEQ ID NO: 4 for preventing or delaying onset of Type 1 diabetes. For example and without limitation the instant specification provides:

- "The present invention is directed to methods, compounds, compositions, combinations, and kits for treating, preventing, suppressing or delaying the onset, or reducing the risk of developing type 1 diabetes, or the symptoms associated with, or related to, type 1 diabetes, in a subject in need thereof. In one aspect, the present invention is directed to compounds, compositions, kits, and methods for endocytic presentation of an immunosuppressive factor for the down regulation of diabetogenic T cells for the treatment or prevention of type 1 diabetes. In yet another embodiment of the present invention, methods, kits, combinations, and compositions containing at least one immunoglobulin, for example, INS, GAD, an insulin protein, a peptide derived from insulin, a diabetogenic epitope, or a T cell receptor engaging determinant, are provided to treat, prevent, suppress, or delay the onset of type 1 diabetes after expression of an IAA predisposition marker." Page 4, lines 11-21.

- "1) A method for the treatment of type 1 diabetes in a patient during the pre-insulitis stage of diabetes by administration of a composition comprising an immunoglobulin or portion thereof linked to a peptide wherein the immunoglobulin or portion thereof is aggregated." Page 6, lines 13-15. "21) The method of paragraph 1 wherein the peptide is selected from the group consisting of GAD1 and GAD2." Page 8, lines 7-8.

- "The present invention is also directed to a methods, kits, combinations, and compositions, comprising: a pharmaceutically-effective amount of an immunoglobulin, or portion thereof, linked to a protein fragment or peptide, wherein the immunoglobulin, or portion thereof, can bind to an Fc receptor. Illustratively, the peptide comprises INSβ, GAD 1, or GAD2." Page 19, lines 1-5.

- "In yet another embodiment of the present invention, the immunoglobulin comprises Ig-INSβ, Ig-GAD1, Ig-GAD2, or an immunoglobulin, or a portion thereof, linked to a peptide, for example a peptide derived from GAD65 or an insulin protein." Page 22, lines 3-5.

- "In yet another embodiment of the present invention, the composition comprises IgINS (peptides derived from human insulin), IgGAD (peptides derived from GAD), IgINSβ, IgGAD1 and IgGAD2." Page 23, lines 2-4.

- "In one embodiment of the present invention, the composition comprises Ig-INSβ, Ig-GAD1, IgGAD2 or an immunoglobulin or a portion thereof linked to a peptide derived from GAD65." Page 24, lines 6-8.

- "In one embodiment of the present invention, a composition is provided comprising an immunoglobulin or portion thereof linked to a protein fragment or peptide wherein the immunoglobulin or portion thereof is capable of binding to an Fc receptor, the peptide being selected from the group consisting of peptides derived from INS and GAD and more specifically INSβ, GAD 1 and GAD2, the composition having the property of being endocytosed by cells bearing the Fc receptor and processed and presented by the cells to present the peptide to endogenous MHC Class II molecules, thereby substantially reducing or preventing activation of diabetogenic T cells specific for the peptide." Page 24, lines 14-21.

- "Other peptides that may be inserted within the variable region within the CDR region of an Ig and utilized for creating compositions for the treatment of type 1 diabetes as taught in the present invention are: GAD1 (Glutamic acid decarboxylase-65 also known as 'GAD65'); corresponding to amino acid residues 524-543 of GAD 65 (Seq. I.D. No. 3 [SRLSKVAPVIKARMMEYGT]) to create chimera Ig-GAD1; and 2) GAD2; corresponding to amino acid residues 206-220 of GAD 65 (Seq. I.D. No. 4 [TYEIAPVFVLLEYVT]); and other peptides derived from GAD65." Page 45, line 20, to page 46, line 2 (bracketed text appears in original).

In view of the above excerpts from the instant specification, Applicants note that the specification as filed clearly provides written support for a method of preventing or delaying onset of Type 1 diabetes comprising administration of an immunoglobulin construct comprising a protein represented by SEQ ID NO: 4.

Further, in the Office Action, the Examiner asserts that the specification "does not teach a peptide consisting of amino acid residues 206-220 of *any* GAD65, e.g., mouse GAD65, rat GAD65, horse GAD65, etc." Applicants note that the claims are to be read in light of the specification. The specification clearly defines (by chemical formula) "amino acid residues 206-220 of GAD65," as a peptide having the chemical formula set forth in SEQ. ID NO 4. Applicants again point to language at page 45, line 20, to page 46, line 2, of the specification as filed reciting: "amino acid residues 206-220 of GAD 65 (SEQ. I.D. No. 4 [TYEIAPVFVLLEYVT])" (bracketed text appears in original specification as filed). Therefore, one of skill in the art reading the specification would immediately recognize that amino acid residues 206-220 of GAD65 are referred to in the specification as SEQ. ID NO 4 which has the amino acid sequence TYEIAPVFVLLEYVT, regardless of the native source of the GAD65 protein or whether it is synthetically produced.

Because the originally filed disclosure would have conveyed to one having ordinary skill in the art that the Applicants had possession of the concept of what is being claimed, the instant written description rejection should be reversed.

IV. Rejection under 35 U.S.C. § 103(a).

Claims 1, 2, 4, 5, 7, 13, 15-19, 22-24, 26 and 28-30 stand rejected under 35 U.S.C. § 103(a) as being unpatentable over WO 98/30706 in view of Chao *et al.* (1999) *PNAS* 96:9299-9304 ("*Chao*"). As will be discussed in detail below, no *prima facie* case of obviousness has been established.

To establish a *prima facie* case of obviousness under 35 U.S.C. § 103, the Office must articulate a reason or rationale that would have prompted a person of ordinary skill in the relevant field to combine the elements in the way the claimed new invention does. See, *e.g.*, *KSR* 550 U.S. 398 (2007); *Omegaflex, Inc. v. Parker-Hannifin Corp.*, 243 Fed. App'x. 592, 595-596 (Fed. Cir. 2007) citing *KSR*. Further, the Supreme Court in *KSR* also stated that that "a court

must ask whether the improvement is more than the predictable use of prior art elements according to their established functions." *KSR* at 1740; emphasis added.

Where the rationale used by the PTO to reject claims as obvious is based on some alleged teaching, suggestion, or motivation in the prior art that would have led one of ordinary skilled in the art to modify the prior art reference or to combine prior art reference teachings to arrive at the claimed invention, the PTO must articulate the following:

(1) a finding that there was some teaching suggestion or motivation, either in the references themselves or in the knowledge generally available to one of ordinary skill in the art, to modify the reference or to combine reference teachings;

(2) a finding that there was a reasonable expectation of success; and

(3) whatever additional findings based on the Graham factual inquiries may be necessary in view of the facts to explain a conclusion of obviousness. See MPEP 2143(G).

According to the Office Action, WO 98/30706 teaches the treatment of autoimmune disorders employing a humanized IgG2b chimeric protein wherein an autoantigen peptide is inserted into the D segment of a CDR3 loop. Office Action at page 9. WO 98/30706 is silent as to GAD65, GAD1 and GAD2. *Chao*, on the other hand, is cited for its disclosure that the GAD65 peptide of SEQ ID NO: 4 is an immunodominant T-cell diabetes antigen in a NOD mouse model. *Chao* at page 9300. The Examiner therefore concludes that it would have been obvious to insert the full length GAD65 protein of *Chao* into a construct of WO 98/30706 and that such a person would have had a reasonable expectation of preventing or delaying the onset of Type 1 diabetes at the priority date of the instant application. As such, the instant obviousness rejection is based on a "teaching, suggestion, or motivation" rationale—so the PTO's argument goes, since GAD65 was a known Type 1 diabetes autoantigen at the time of filing, one of skill in the art would have been motivated to insert it into the construct of WO 98/30706 and would have had a reasonable expectation of preventing or delaying the onset of Type 1 diabetes.

A. No reasonable expectation of success.

Even if one of ordinary skill in the art would have had some reason combine the construct of WO 98/30706 with the GAD65 peptide (SEQ ID NO: 4) of *Chao* to prevent or delay the onset of type 1 diabetes, which is not admitted, such a person would not have had a reasonable

expectation of success in preventing or delaying the onset of type 1 diabetes, particularly in a subject that had undergone insulin autoantibody seroconversion.

The Examiner relies on WO 98/30706 which discloses a fusion protein having the proteolipid protein (PLP) autoantigen inserted into the D segment of a CDR3 loop. PLP is an autoantigen associated with multiple sclerosis. Applicants respectfully submit that the multiple sclerosis test model used in WO 98/30706 (experimental allergic encephalomyelitis) is far different from the type 1 diabetes NOD mouse model used in examples within the instant application such that any success or failure shown in WO 98/30706 would not be at all predictive of success or failure of an Ig-GAD2 fusion protein in prevention or delay of type 1 diabetes as presently claimed.

Specifically, the relevant examples in WO 98/30706 (*e.g.* Examples I and XI) involve induction of an immune response with a known pathogenic peptide (PLP1) followed by treatment of the induced immune response with a slightly altered version of the very same peptide (PLP-LR) introduced in the form of a chimeric antibody immunomodulating agent. PLP-LR is an analog of PLP1 in which Trp144 and His147 are replaced with Leu and Arg, respectively. Therefore, in Examples I and XI of WO 98/30706, a disease state is induced with a known pathogenic peptide and then treated with a slightly altered non-pathogenic version of the very same peptide.

In stark contrast to those examples, the onset of type 1 diabetes in the NOD mouse model is a *spontaneous* event not triggered by administration of a known peptide antigen. Because no inducer peptide is known or administered, it was completely unpredictable at the time the present invention was made which peptide antigen, if any, when incorporated into compositions disclosed in the instant application, would have any impact on type 1 diabetes, let alone delay or prevent that disease state. This is very different from the situation in WO 98/30706 in which the disease inducing peptide was known at the outset, and treatment was provided with a slight variation of the very same inducer peptide. In view of these significant differences and the highly unpredictable area of art of the presently claimed invention, a person of ordinary skill in the art at the time the present invention was made would not have had a reasonable expectation of success in delaying or preventing type 1 diabetes according to the presently claimed methods. Applicants respectfully submit that the outcome of the presently claimed methods was highly unpredictable at the time the present invention was made.

Furthermore, one of ordinary skill in the art at the time the present invention was made would not have had a reasonable expectation that the GAD65 peptide of *Chao* (SEQ ID NO: 4), selected from the numerous type 1 diabetes autoantigen peptides and protein fragments known or suspected at the time, would prevent or inhibit diabetes as presently claimed. There is no articulated rationale in the record for selection of any particular diabetogenic peptide or protein fragment, nor any indication why a person of ordinary skill in the art would have had a reasonable expectation of delaying or preventing type 1 diabetes in an IAA positive subject with any such peptide or protein fragment.

For at least the foregoing reasons, a person of ordinary skill in the art would not have had a reasonable expectation of success of preventing or delaying the onset of diabetes according to the presently claimed methods. Withdrawal of the instant rejection is therefore respectfully requested.

B. Unexpected results.

Moreover, it is well settled that "[o]ne way for a patent applicant to rebut a *prima facie* case of obviousness is to make a showing of 'unexpected results,' *i.e.*, to show that the claimed invention exhibits some superior property or advantage that a person of ordinary skill in the relevant art would have found surprising or unexpected." *In re Soni*, 54 F.3d 746, 750 (Fed. Cir. 1995). Additionally, as was articulated in *In re Soni*, "when an applicant demonstrates *substantially* improved results…and states that the results were unexpected, this should suffice to establish unexpected results in the absence of evidence to the contrary. 54 F.3d 746 at 751(Fed. Cir. 1995).

As is discussed in detail below, the presently claimed soluble Ig-GAD2 composition provides unexpected results in that it has the ability to rescue residual and form new insulin-producing β cells. Applicants point to an article co-authored by inventors Zaghouani and Gregg (Jain *et al.*, Innocuous IFNγ induced by adjuvant-free antigen restores normoglycemia in NOD mice through inhibition of IL-17 production, JEM Vol. 205, No. 1 (2008); hereinafter "*Jain*") and declaration under 37 CFR 1.312 of Habib Zaghouani dated July 15, 2009 ("Zaghouani Declaration II"), both provided herewith, setting forth unexpected properties of the soluble Ig-GAD2 construct.

Applicants respectfully direct the Examiner's attention to page 209 of *Jain* (section titled "Treatment with Ig-GAD2 increases the number of healthy pancreatic islets"). It will be noted that animals treated with the soluble Ig-GAD2 construct had significantly greater number of total islets than the hyperglycemic or diabetic mice (P = 0.0001). *Jain*, Figures 3B and 3 C. The number of insulin-positive islets also increased from 14 per pancreas at the prediabetic (hyperglycemic) stage to 29 per pancreas upon treatment with Ig-GAD2. Also, the 15-week treatment group had a higher number of islets with periinsulits (38% vs. 30%) or no insulitis (35% vs. 17%) relative to the hyperglycemic stage. *Jain*, Fig 3C. Overall, the treatment with Ig-GAD2 led to a significant increase in the number of non-inflamed (healthy) islets.

Moreover, as set forth starting at column 2, page 210 of *Jain*, an experiment was conducted to determine whether the increased number of healthy islets in the treated animals was caused by regression of cell infiltration and/or formation of new β cells. To address this question, treated mice were injected with 100 mg/kg of the proliferation indicator BrdU and pancreas sections were double stained with anti-insulin and BrdU antibodies and analyzed for BrdU incorporation and insulin production. Because the relevant figures in *Jain* for this experiment include colored arrows, Applicants attach herewith for the Examiner's convenience annotated versions of these figures with labels indicating colors of the arrows. Blue arrows indicated $BrdU^+$ cells, green arrows indicate $insulin^+$ cells, and red arrows indicated $BrdU^+/Insulin^+$ cells.

Applicants point out that BrdU staining was visible in the highly proliferative luminal intestinal cells used as a control, but these had no staining with anti-insulin antibody. Fig. 4A, blue arrow. Islets of nondiabetic 5-wk old mice were positive for insulin, but did not incorporate BrdU, suggesting that these insulin-producing β cells were not dividing. Fig. 4B, green arrow. Thus, under normal circumstances, insulin production emanates from existing β cells whose nuclei do not incorporate BrdU, giving a minimal number of BrdU/insulin double positive β cells ($BrdU^+/insulin^+$). The hyperglycemic mice showed very few insulin-producing β cells and no BrdU incorporation (Fig. 4 C, green arrow), resulting in an insignificant number of $BrdU^+/insulin^+$) β cells (Fig. 4 E). In contrast, as shown in Fig. 4 D, islets from the 25-wk treatment group showed $insulin^+$ β cells that were either BrdU- (green arrows; residual β cells) or $BrdU^+$ (red arrows; newly formed β cells). The number of insulin-producing dividing β cells

was significantly (P = 0.0001 for treated group compared to hyperglycemic group) increased in all 10 mice in which treatment restored normoglycemia. Fig. 4 E. Error bars indicated the standard deviation of 10 pacreata.

Overall, Applicants submit that these results indicate that treatment with soluble Ig-GAD2 reduces cell infiltration, leading to rescue of residual and formation of new insulin producing β cells. Thus, even if the Examiner has demonstrated a *prima facie* case of obviousness, which Applicants do not admit, the unexpected results obtained with soluble Ig-GAD2 would overcome such a rejection of the instant claims. Accordingly, withdrawal of the instant rejection is respectfully requested.

C. Each and every claim limitation not disclosed in the prior art.

Claim 1 and all claims depending there from specify that "the subject has undergone insulin autoantibody seroconversion prior to the administering step." This limitation is simply not disclosed in the prior art of record. Because the prior art when combined does not teach each and every limitation of the instantly claimed invention, the asserted prima facie case of obviousness fails.

For at least the foregoing reasons, no *prima facie* case of obviousness has been established and reversal of the instant obviousness rejection is respectfully requested.

CONCLUSION

The application is believed to be in condition for allowance. Early and favorable considerations is respectfully requested. The Commissioner is hereby authorized to charge deposit account 02-1818 for any fees which are due and owing.

Respectfully submitted,

K&L GATES LLP

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Dated: May 20, 2011

Enclosures:

Jain *et al.*, JEM Vol. 205, No. 1 (2008).
Declaration under 37 CFR 1.312 of Habib Zaghouani dated July 15, 2009.

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JEM

ARTICLE

Innocuous IFNγ induced by adjuvant-free antigen restores normoglycemia in NOD mice through inhibition of IL-17 production

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The role of Th17 cells in type I diabetes (TID) remains largely unknown. Glutamic acid decarboxylase (GAD) sequence 206–220 (designated GAD2) represents a late-stage epitope, but GAD2-specific T cell receptor transgenic T cells producing interferon γ (IFNγ) protect against passive TID. Because IFNγ is known to inhibit Th17 cells, effective presentation of GAD2 peptide under noninflammatory conditions may protect against TID at advanced disease stages. To test this premise, GAD2 was genetically incorporated into an immunoglobulin (Ig) molecule to magnify tolerance, and the resulting Ig-GAD2 was tested against TID at different stages of the disease. The findings indicated that Ig-GAD2 could not prevent TID at the preinsulitis phase, but delayed TID at the insulitis stage. More importantly, Ig-GAD2 sustained both clearance of pancreatic cell infiltration and β-cell division and restored normoglycemia when given to hyperglycemic mice at the prediabetic stage. This was dependent on the induction of splenic IFNγ that inhibited interleukin (IL)-17 production. In fact, neutralization of IFNγ led to a significant increase in the frequency of Th17 cells, and the treatment became nonprotective. Thus, IFNγ induced by an adjuvant free antigen, contrary to its usual inflammatory function, restores normoglycemia, most likely by localized bystander suppression of pathogenic IL-17–producing cells.

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Abbreviations used: GAD, glutamic acid decarboxylase; HEL, hen egg lysozyme; IAA, insulin autoantibody; TID, type I diabetes.

Antigen-specific approaches have been defined that could prevent the development of type I diabetes (TID; for review see [1]). However, antigen-driven strategies that could counter the disease at more advanced stages have yet to be defined (1). As with many autoimmune disorders, TID most likely involves multiple autoantigens and diverse T cell specificities (2, 3). In addition, sequential spreading seems to orchestrate TID, with insulin being required for the initiation of the disease (4), whereas GAD-reactive T lymphocytes are more involved at later stages of TID (5, 6). Thus, for an antigen-specific therapy to be effective and practical against TID, it would have to target late-stage epitopes that could counter diverse aggressive T cell specificities. GAD2 peptide corresponding to amino acid sequence 206–220 of GAD is considered a late-stage epitope because its T cell reactivity is detected at an advanced stage of the disease (7). TCR transgenic T cells specific for GAD2 peptide were generated, but these produced both IFNγ and IL-10 and were protective against TID when tested in a transfer model of passive diabetes (8). Given this information, we reasoned that effective presentation of GAD2 peptide in vivo under noninflammatory conditions would possibly induce IFNγ- and IL-10–producing T cells that could protect against TID. Because IFNγ displays inhibitory activity against Th17 cells (9, 10), the approach could prove effective even at an advanced stage of the disease if Th17 cells play a pathogenic role in TID. To test these premises, GAD2 peptide was genetically inserted into the variable region of a heavy chain Ig gene, and the fusion gene was transfected into

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a myeloma B cell line along with the parental light chain gene for expression as a complete Ig-GAD2. Because Igs internalize into APCs via Fcγ receptor (FcγR), the grafted GAD2 peptide will be efficiently dragged into the cells, where it accesses newly synthesized MHC class II molecules, and presentation will be significantly increased relative to free peptide, as was the case for other diabetogenic and encephalitogenic peptides (11–16). Moreover, because Igs are self-proteins, when injected into animals, presentation occurs without inflammation, leading to lack of costimulation and magnification of tolerance (12–14).

In an initial attempt, Ig-GAD2 was tested for prevention of TID before insulitis, but proved ineffective for delay of disease. However, when the treatment was administered at the insulitis stage, protection against TID was observed. More importantly, Ig-GAD2 given to hyperglycemic mice at the prediabetic stage was highly effective, leading to clearance of pancreatic cell infiltration, stimulation of β-cell division, and restoration of normoglycemia. Investigation of the mechanism underlying reversal of disease revealed the presence of splenic IFNγ-producing GAD2-specific T cells that were, indeed, responsible for reversal of disease because neutralization of IFNγ restored progression to overt diabetes. In parallel, the protected mice had reduced production of IL-17 cells in the spleen and pancreas relative to diabetic mice, and exogenous IL-17 reinstated progression to diabetes in the otherwise protected animals. Thus, splenic IFNγ likely interferes with supply of Th17 to the pancreas, leading to clearance of islet infiltration, stimulation of β cell division, and restoration of normoglycemia.

RESULTS

Treatment with Ig-GAD2 restores normoglycemia

The I-A^{g7}–restricted diabetogenic GAD2 peptide was genetically expressed on an Ig molecule, and the resulting Ig-GAD2 was used to test against TID. Similarly, the nondiabetogenic I-A^{g7}–restricted hen egg lysozyme (HEL) 11–25 sequence was also incorporated in an Ig, and the resulting Ig-HEL was used as control (16). The chimeras were then tested for presentation to a GAD2-specific T cell line. As indicated in Fig. 1, Ig-GAD2 was taken up by APCs, processed, and presented to GAD2-specific T cells much more efficiently than free GAD2 peptide (Fig. 1 A, B). The control Ig-HEL was unable to induce similar stimulation of the GAD2-specific T cells. Ig-GAD2 was then assayed for tolerogenic function by testing for prevention of TID in young NOD mice undergoing the initial phase of islet infiltration, which is referred to as the preinsulitis stage. The results in Fig. 1 C indicate that Ig-GAD2 had no significant long-term protective effect against TID relative to Ig-HEL or untreated mice. Knowing that insulin, but not GAD, is required for initiation of diabetes at the preinsulitis stage (4), the lack of protection might have been caused by the absence of activated GAD2-specific target T cells at this stage. We then tested Ig-GAD2 for suppression of diabetes at a later stage during insulitis. It has been shown that seroconversion to insulin autoantibody (IAA) production is indicative of ongoing insulitis (17, 18), and our own studies indicated that among the 83% of female NOD mice that seroconvert to IAA at the age of 8–11 wk, 84% develop overt

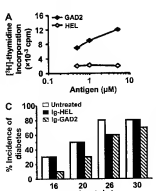

Figure 1. Ig-GAD2 treatment given at the insulitis-positive stage reverses T1D. (A and B) Presentation of Ig-GAD2 to T cells. NOD splenic APCs were incubated with free peptides (A) or Ig chimeras (B), and 1 h later GAD2-specific T cells were added. Activation was assessed by [³H]thymidine incorporation. HEL peptide and Ig-HEL were included as negative controls. (C and D) Percentage of mice free of diabetes upon treatment with Ig-GAD2 or the control Ig-HEL at the preinsulitis (C) and insulitis (D) stage, respectively. All mice were monitored for blood glucose from 12 to 30 wk of age. *, $P < 0.05$; **, $P < 0.01$ compared with untreated mice. A mouse is considered diabetic when blood glucose level is ≥ 300 mg/dl for two consecutive weeks. An untreated group was included in all experiments for comparison purposes. At least 10 mice were included in each experimental group.

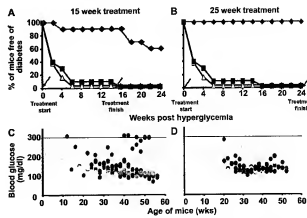

Figure 2. Ig-GAD2 treatment given at the prediabetic stage reverses T1D. (A and B) Percentage of mice free of diabetes upon treatment with Ig-GAD2 or the control Ig-HEL at the hyperglycemic stage for 15 (A) or 25 wk (B). Arrows indicate the beginning and end of treatment. (C and D) Individual blood glucose levels of Ig-GAD2 treated mice are shown from the week of diagnosis of hyperglycemia up to 52 or 56 wk of age for 15 (C) and 25 wk (D) treatment regimens. Each dot represents a different mouse. A mouse is considered hyperglycemic or diabetic when blood glucose level is between 160–250 or ≥ 300 mg/dl for two consecutive weeks, respectively. The shaded area indicates the hyperglycemic range of blood glucose levels and the line depicts the diabetic level. An untreated group was included in all experiments for comparison purposes. At least 10 mice were included in each experimental group.

diabetes (16). Ig-GAD2 was then tested for delay of T1D upon IAA-seroconversion. An initial regimen consisting of 300 μg of Ig-GAD2 at week 1, 2, and 3 upon IAA seroconversion indicated that 50% of mice were protected against diabetes up to 30 wk of age (unpublished data). This was promising, as the same regimen did not protect at the preinsulitis stage, and it prompted us to test a prolonged regimen for suppression of diabetes. As indicated in Fig. 1 D, administration of Ig-GAD2 into insulitis-positive (IAA^+) mice delayed T1D, and most of the animals (7 out of 10) remained free of disease by week 30 of age. Ig-HEL–treated animals, like the untreated group, were not significantly protected (Fig. 1 D). These results indicate that Ig-GAD2 protects against T1D at a later, rather than earlier, stage of the disease. We then evaluated Ig-GAD2 at the more advanced hyperglycemic stage. Accordingly, blood glucose levels were monitored beginning at week 12 of age, and mice displaying hyperglycemia between the ages of 14 to 30 wk were subjected to a daily injection of Ig-GAD2 for 5 d, and then a weekly injection for either 15 or 25 wk. The results show that 90% of the mice under the 15-wk Ig-GAD2 regimen were protected against diabetes throughout the 15 wk of treatment (Fig. 2 A). However, only 60% of the mice remained disease-free for the 10 wk after cessation of treatment. Untreated and Ig-HEL recipient mice became diabetic by the fifth week of hyperglycemia. When the regimen was extended to 25 wk, 100% of the Ig-GAD2–treated animals were protected (Fig. 2 B), and normoglycemia was restored in all mice. This status persisted throughout the duration of the study (mice aged 52–56 wk). The weekly blood glucose level of individual mice shows a consistent pattern of return to normoglycemia for 6 out of 10 mice in the 15-wk treatment regimen, and all 10 animals in the 25-wk regimen (Fig. 2, C and D). A detailed description of the day of onset, as well as the level of blood glucose at the beginning and termination of the hyperglycemic treatment regimen, is provided in Table I. These results demonstrate that protection against the disease by Ig-GAD2 occurs at the onset of insulitis, whether this manifests at an early or an older age. Overall, this antigen-specific single-epitope therapy by Ig-GAD2 restores normoglycemia in prediabetic mice, a stage at which GAD2-specific T cells could be targeted.

Treatment with Ig-GAD2 increases the number of healthy pancreatic islets

To determine whether the restoration of normoglycemia by Ig-GAD2 is caused by interference with cell infiltration, the mice were subjected to histopathologic analysis upon

Table I. Blood glucose (BG) level at the onset of hyperglycemia and at termination of treatment regimen[a]

Mouse ID	Age at onset of hyperglycemia (wk)	Blood glucose level before treatment (mg/dl)[b]	Blood glucose level after termination of treatment (mg/dl)[c]
15-wk treatment regimen			
106.1	30	161	133
106.7	30	165	113
119.2	30	180	285[d]
119.8	26	182	112
119.9	28	165	128
190.1	12	250	118
191.2	18	179	99
192.2	26	212	134[d]
192.3	16	160	308[d]
196.1	22	222	150[d]
25-wk treatment schedule			
220.1	26	160	135
221.12	24	174	120
206.2	24	231	121
203.1	24	181	110
232.3	20	173	140
236.6	14	185	116
225.9	26	163	120
237.8	20	250	130
227.7	28	180	137
244.4	16	171	123

[a]The results illustrated in this table were from the mice treated with either the 15- or 25-wk Ig-GAD2 treatment regimen and described in Fig. 2. The hyperglycemia onset represent the week during which the mice showed, for the first time, a blood glucose level of 160–250 mg/dl.
[b]Blood glucose level obtained the second week of hyperglycemia.
[c]Blood glucose level obtained at the termination of treatment regimen.
[d]Mice that became diabetic before or within 7 wk of treatment termination.

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completion of the treatment regimen. As indicated in Fig. 3, most of the islets in hyperglycemic and diabetic mice exhibited intrainsulitis (Fig. 3 A, 1, 2, and 3), the majority of islets in treated mice were not inflamed (Fig. 3 A, 4) or had only mild periinsulitis (Fig. 3 A, 5 and 6). Moreover, enumeration of the islets indicated that the treated animals had a significantly greater number of total islets than the hyperglycemic or diabetic mice (Fig. 3 B). The number of insulin-positive islets also increased from 14 per pancreas at the prediabetic (hyperglycemic) stage to 29 per pancreas upon treatment with Ig-GAD2. Also, the 15-wk group had a higher number of islets with periinsulitis (38 vs. 30%) or no insulitis (35 vs. 17%) relative to the hyperglycemic stage (Fig. 3 C). On the other hand, the number of islets with severe and mild intrainsulitis were reduced in the treated versus hyperglycemic mice (8 and 19% vs. 22 and 31%, respectively). Surprisingly, in the 25-wk treatment group, although the total number of islets was reduced to that of the hyperglycemic stage, most of these islets exhibited no insulitis (60%), periinsulitis (28%), or mild intrainsulitis (12%; Fig. 3 C). Overall, the treatment with Ig-GAD2 led to a significant increase in the number of noninflamed (healthy) islets.

Ig-GAD2–treated mice exhibit pancreatic β-cell division

The increase in the number of healthy islets in the treated mice could be caused by regression of cell infiltration and/or formation of new β cells. To address this premise, the treated mice were injected with the proliferation indicator BrdU and killed, and pancreatic sections were double stained with anti-insulin and -BrdU antibodies and analyzed for BrdU incorporation

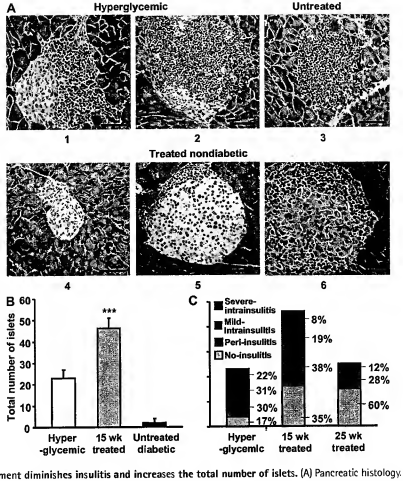

Figure 3. Ig-GAD2 treatment diminishes insulitis and increases the total number of islets. (A) Pancreatic histology. Four sections per pancreas (8 μm thick each cut 100 μm apart) from 5 hyperglycemic (A, 1 and 2), untreated diabetic (A, 3) or 15-wk Ig-GAD2–treated nondiabetic (A, 4, 5, and 6) mice were stained with hematoxylin and eosin and analyzed at 400x magnification. For the hyperglycemic and untreated diabetic mice sections were made the second week of diagnosis. For the treated nondiabetic mice, histology was performed 7 wk after the last treatment. (B) Total islets per pancreas as determined by hematoxylin and eosin staining from the three groups of mice described in A. Only structures with visible islet cells and incomplete infiltration were counted. (C) Islets from hyperglycemic, 15 and 25 wk Ig-GAD2–treated mice were scored as described in Materials and methods, and the percentages represent the number of islets of a given score over the total number of islets from B. The sections were made at the time indicated in A, and 2 d after the last Ig-GAD2 injection for the 25 wk–treated group. ***, $P = 0.0001$ for the total number of islets in 15 wk–treated versus hyperglycemic group. Error bars indicate the SD. Bars, 25 μm.

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and insulin production. BrdU staining was visible in the highly proliferative luminal intestinal cells used as control, but these had no staining with anti-insulin antibody (Fig. 4 A). Islets of nondiabetic 5-wk-old mice were positive for insulin, but did not incorporate BrdU, suggesting that these insulin-producing β cells were not dividing (Fig. 4 B). Thus, under normal circumstances, insulin production emanates from existing β cells whose nuclei do not incorporate BrdU, giving a minimal number of BrdU/insulin double-positive ($BrdU^+$/$insulin^+$) β cells (Fig. 4 E). The hyperglycemic mice showed very few insulin-producing β cells and no BrdU incorporation (Fig. 4 C), resulting in an insignificant number of $BrdU^+$/$insulin^+$ cells (Fig. 4 E). In contrast, islets from the 25-wk treatment group showed $insulin^+$ β cells that were either $BrdU^-$ (residual β cells) or $BrdU^+$ (newly formed β cells; Fig. 4 D). Notably, the number of these insulin-producing dividing β cells was significantly increased in all 10 mice in which treatment restored normoglycemia (Fig. 4 E). Interestingly, the total number of dividing cells producing insulin ($BrdU^+$/$insulin^+$) was low, and it may not solely account for the restoration of normoglycemia. $BrdU^-$/$insulin^+$ residual islet cells, which amounted to 81 cells per pancreas, may have also contributed to the control of blood glucose levels, and these likely represent a combination of newly formed and residual β cells that were rescued by regression of infiltration. There was a minimal number of dividing β cells ($BrdU^+$/$insulin^+$) in the normal and hyperglycemic groups, despite the presence of 927 and 50 $BrdU^-$/$insulin^+$ β cells, respectively. These results indicate that treatment with Ig-GAD2 reduces cell infiltration, leading to rescue of residual and formation of new β cells.

Ig-GAD2–treated mice produce protective IFNγ

Previous studies indicated that Ig-GAD1, which is an Ig chimera carrying GAD524–543, and Ig-INSβ carrying insulin 9–23 aa residues induce T regulatory (T reg) cells and prevent T1D only when given in an aggregated, but not soluble, form (15, 16). This is because aggregated, but not soluble, Ig chimeras cross-link FcγR on APCs, induce IL-10 by the presenting cells, and expand T reg cells (15, 16). In this study, only soluble Ig-GAD2 was used for treatment. Despite the fact that soluble Ig-GAD2 does not induce the production by APCs of the T reg cell growth factor IL-10 (19) and is predicted not to expand T reg cells, it was tested for expansion of T reg cells in hyperglycemic mice before and after treatment with Ig-GAD2. The results indicated that the percentage of $CD4^+CD25^+CD62L^+$ and $CD4^+CD25^+FoxP3^+$ T cells in the spleen, as well as in the pancreatic lymph nodes, were similar before and after treatment (Table II). This suggests that T reg cells play a minimal role in disease reversal by soluble Ig-GAD2.

For Ig-GAD2 therapy, it is logical to contemplate that the resolution of the inflammatory infiltration is caused by modulation of GAD2-reactive diabetogenic T cells. Because T1D is likely to involve multiple autoantigens, the restoration of normoglycemia would require modulation of diverse T cell specificities. Thus, the plausible hypothesis postulates that

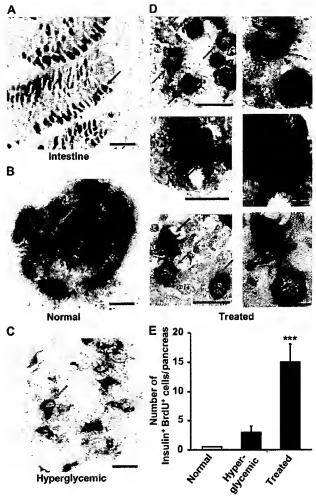

Figure 4. β Cells from mice treated with Ig-GAD2 incorporate BrdU. Mice (10 per group) were given 100 mg/kg BrdU i.p. and killed 3 h later. Sections of the small intestine or pancreas were stained with anti-insulin and -BrdU antibodies, and then analyzed for insulin production (blue cytoplasmic rim) and BrdU incorporation (red nuclei) at 400× magnification. Blue arrows indicate $BrdU^+$ cells, green arrows indicate $insulin^+$ cells, and red arrows indicate $BrdU^+$/$insulin^+$ cells. Intestinal lumen (A) and β cells (D) from mice recipient of the 25-wk Ig-GAD2 regimen. (B) Beta cells from 5-wk-old nondiabetic NOD mice. (C) β Cells from hyperglycemic mice. (E) Total number of $insulin^+$/$BrdU^+$ cells in nondiabetic (normal), hyperglycemic, and Ig-GAD2–treated nondiabetic NOD mice. ***, $P = 0.0001$, treated group compared with hyperglycemic group. Error bars indicate the SD of 10 pancreata. Bars: (A–C) 25 μm; (D, left) 20 μm; (D, right) 5 μm D.

recovery from the disease involved localized bystander suppression. To test this premise, the splenic cells from recovered mice were stimulated with GAD2 peptide and assessed for both suppressive and inflammatory cytokines. The results indicate that although no measurable IL-4 or TGFβ was detected (not depicted), there was significant IFNγ and IL-10 production by these cells relative to the control HEL peptide (Fig. 5 A). Moreover, intracellular cytokine analysis of CD4 and Vβ8.2

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Table II. Reversal of T1D by Ig-GAD2 does not significantly increase expression of phenotypic markers associated with T reg cells[a]

	$CD4^+CD25^+FoxP3^+$		$CD4^+CD25^+CD62L^+$	
	Untreated	Treated	Untreated	Treated
SP	4.9	3.3	2.0	1.9
PLN	5.0	5.5	3.0	4.4

[a]Spleen and pancreatic lymph node cells from Ig-GAD2–treated and control untreated mice were stained with anti-CD4, -CD25, and -CD62L or -FoxP3 antibody. The cells were gated on $CD4^+$ cells and analyzed for CD25, FoxP3, and CD62L expression by flow cytometry.

T cells indicated that the majority of the T cells produced only IFNγ, with fewer cells stained positive for both IL-10 and IFNγ (Fig. 5 B). Indeed, upon stimulation with GAD2 peptide, a significant increase (four- to sevenfold) in the number of CD4/Vβ8.2 T cells producing IFNγ was observed in the Ig-GAD2–treated versus untreated mice. Because IL-10 is known for its anti-Th1 suppressive function (20–22), we suspected that protection against the disease involves the function of these IL-10/IFNγ–producing cells. To our surprise, however, when in vivo cytokine neutralization was performed along with Ig-GAD2 treatment, the recovery persisted with anti–IL-10 treatment, but was nullified by removal of IFNγ (Fig. 5 C). Isotype-matched rat IgG had no effect on the disease (Fig. 5 C). These observations indicate that IFNγ, contrary to its well-defined inflammatory function, is likely involved in modulation of inflammation and restoration of normoglycemia.

Ig-GAD2 treatment interferes with IL-17 production in an IFNγ-dependent fashion

Th17 cells represent a newly defined subset of pathogenic T cells whose development can be facilitated by TGFβ and

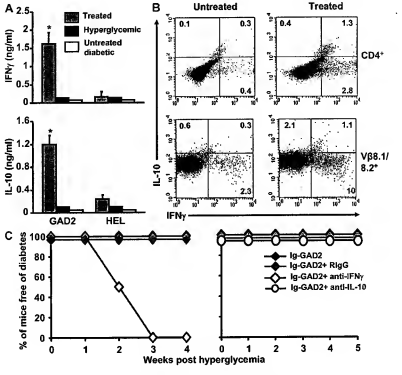

Figure 5. Treatment with Ig-GAD2 induces IFNγ that sustains protection against diabetes. (A) Splenocytes from hyperglycemic mice recipient of the 25-wk Ig-GAD2 treatment regimen were stimulated in vitro with GAD2 and the control HEL peptide and IFNγ and IL-10 were measured by ELISA as described in Methods. Diabetic as well as untreated hyperglycemic mice were included for control purposes. Each bar represents the mean ± SD of three independent experiments. *, $P = 0.01$ when stimulation by GAD2 is compared with HEL peptide. (B) Intracellular IL-10 and IFNγ production by splenic CD4 (top) or Vβ8.1/8.2 (bottom) T cells from the 25-wk–treated (right) and hyperglycemic untreated (left) mice. This was done by intracellular staining upon stimulation with GAD2 peptide, as indicated in the Materials and methods. Data are representative of three independent experiments. (C) Percentage of mice free of diabetes upon in vivo neutralization of IFNγ (left) or IL-10 (right) during treatment with Ig-GAD2 at the hyperglycemic stage. Anti-IFNγ (R4-6A4), anti-IL-10 (JES5-2A5), or isotype control rat IgG were given to mice (500 μg/mouse per injection) i.p. every 3 d for 4 consecutive weeks, beginning with the first injection of Ig chimeras. The mice received a total of nine antibody injections. At least eight mice were included in each experimental group.

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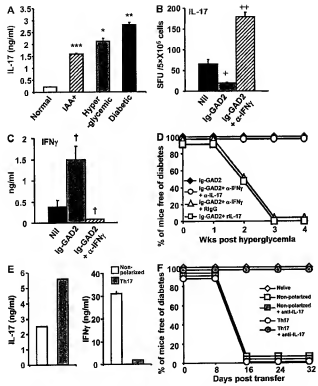

Figure 6. Neutralization of IFNγ during treatment with Ig-GAD2 restores IL-17 production. (A) IL-17 response from the splenocytes of preinsulitis (normal), insulitis-positive (IAA+), hyperglycemic, and diabetic mice upon in vitro stimulation with anti-CD3 antibody. Data are representative of three independent experiments. ***, P = 0.0004, insulitis-positive versus normal; *, P = 0.02, hyperglycemic versus insulitis-positive; **, P = 0.005, diabetic versus hyperglycemic group. IL-17 (B) and IFNγ (C) response from splenocytes of mice recipient of anti-IFNγ during treatment with Ig-GAD2 at the hyperglycemic stage. Splenocytes were harvested when the mice became diabetic on the fourth week of treatment; they were stimulated in vitro with GAD2 peptide, and their responses were measured by ELISPOT and ELISA, respectively. Nil (diabetic) and Ig-GAD2–treated groups were included as controls. Data are representative of three independent experiments. †, P = 0.01, treated versus nil group; ††, P = 0.001, Ig-GAD2 + anti-IFNγ versus Ig-GAD2 group. †, P = 0.04, Ig-GAD2 versus nil group; †, P = 0.02, Ig-GAD2 + anti-IFNγ versus Ig-GAD2 group. (D) Percentage of mice free of diabetes upon administration of recombinant IL-17 or neutralization of both IFNγ and IL-17 during treatment with Ig-GAD2 at the hyperglycemic stage. IL-17 was administered (1 μg/mouse per injection) i.p. daily for 5 consecutive days, beginning with the first injection of Ig-GAD2. Subsequently, the mice received an injection of rIL-17 every week, along with Ig-GAD2. An injection of anti-IFNγ (R4-6A4; 500 μg/mouse) and anti-IL-17 (TC11-18H10; 200 μg/mouse) was given on the first day of treatment with Ig-GAD2 after diagnosis of hyperglycemia. Four additional injections were given at 4-d intervals. At least eight mice were included in each experimental group. (E and F) Th17-polarized cells induce diabetes. (E) IL-17 (left) and IFNγ (right) responses from the nonpolarized and Th17 polarized splenocytes were measured by ELISA. Each bar represents the mean ± SD of triplicate wells. (F) Percentage of mice free of diabetes upon adoptive transfer of 10×10^6 naive, nonpolarized and Th17-polarized cells in NOD.scids (4–6 wk old). Additional groups received IL-17 neutralizing antibody, along with Th17-polarized and nonpolarized cells for control purposes. Anti–IL-17 antibody (TC11-18H10; 200 μg/mouse) was given on the day of transfer, and two additional injections were given at day 4 and 16 after transfer.

IL-6 or interfered with by IFNγ or IL-27 (9, 10, 23–26). Because Ig-GAD2 treatment induces IFNγ, we sought to determine whether restoration of normoglycemia involves interference with IL-17 production. Accordingly, we began by assessing whether IL-17 is produced by NOD T cells, and followed the pattern of its secretion during disease progression. Fig. 6 A shows that stimulation with anti-CD3 antibody did not induce measurable IL-17 by splenocytes from normal 4-wk old mice. However, IL-17 was evident upon IAA-seroconversion and increased significantly when the mice progressed to hyperglycemia and diabetes. The treatment with Ig-GAD2 at the hyperglycemic stage significantly reduced the frequency of GAD2-specific IL-17–producing cells as measured by spot formation (Fig. 6 B). However, neutralization of IFNγ by administration of anti-IFNγ antibody along with Ig-GAD2 restored even higher frequency of Th17 cells. This Th17 restoration is likely caused by complete neutralization of IFNγ because IFNγ-producing Th1 cells could not be detected by ELISPOT (not depicted) and no measurable IFNγ cytokine was found by ELISA (Fig. 6 C). It is thus likely that the restoration of diabetes by neutralization of IFNγ during treatment with Ig-GAD2 (Fig. 5 C) is caused by restoration of Th17. In fact, administration of rIL-17 along with Ig-GAD2 treatment nullifies tolerance and restores diabetes (Fig. 6 D). Moreover, administration of both anti-IFNγ and –IL-17, but not anti-IFNγ and rat IgG, simultaneously protects against T1D (Fig. 6 D), further confirming the interplay between IFNγ and IL-17. To ensure that Th17 cells can be diabetogenic, we chose the BDC2.5 TCR transgenic T cells (27) for polarization with anti-CD3 and -CD28 antibodies and tested for transfer of diabetes into NOD.scid mice. The rationale for this choice instead of Ig-GAD2–induced Th17 cells lies in the fact that the BDC2.5 cells are well characterized and represent a homogeneous population in which the number of cells to be transferred can be precisely controlled. In addition, the Ig-GAD2/NOD model represents a polyclonal system in which the different subsets of T cells cannot be separated. Thus, BDC2.5 T cells were stimulated with anti-CD3 and -CD28 antibodies in the presence or absence of Th17 polarizing factors, and the cells were tested for transfer of diabetes into NOD.scid mice.

As indicated in Fig. 6 E, the Th17-polarized cells had enhanced levels of IL-17 compared with nonpolarized cells, but no measurable IFNγ, whereas nonpolarized cells produced significant IFNγ. These results indicate that the polarization to Th17 was significant under the chosen conditions. Furthermore, when the polarized cells were transferred into NOD.scid mice, diabetes manifested within 16 d after transfer, as with activated, but not polarized, T cells (Fig. 6 F). Diabetes did not occur when the transfer was made with naive BDC2.5 cells. In addition, when IL-17 was neutralized by injection of anti–IL-17

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antibody in the mice recipient of Th17-polarized cells, the disease did not manifest. However, neutralization of IL-17 did not protect against diabetes transferred by nonpolarized cells. These results indicate that Th17 cells producing IL-17 can transfer diabetes into naive mice. The results are thus interpreted to indicate that Ig-GAD2 mobilizes IFNγ-producing splenic Th1 cells that interfere with IL-17–producing diabetogenic lymphocytes to reduce inflammation, sustain islet formation, and restore normoglycemia.

Treatment with Ig-GAD2 sustains long-lasting production of IFNγ in the spleen and nullifies IL-17 in the pancreas

At the hyperglycemic stage, most of the pathogenic T cells likely reside in the pancreas as differentiated cells that have already been exposed to antigen (28, 29). Because IFNγ has been suggested to interfere with the differentiation of naive cells into Th17 (9, 10), it is likely that IFNγ Th1 cells operate their interference with Th17 in the spleen or pancreatic lymph nodes rather than the pancreas. Analysis of the dynamics of both populations at the beginning, as well as at the end, of Ig-GAD2 treatment indicated that during the initial phase of hyperglycemia, IFNγ-producing Th1 cells are mostly located in the spleen (Fig. 7 A), whereas Th17 cells reside in the pancreas (Fig. 7 B). However, at the end of the treatment, Th1 cells remain in the spleen (Fig. 7 C), whereas Th17 cells are undetectable in any organ (Fig. 7 D). These results suggest that Ig-GAD2 induces IFNγ in the spleen, which likely interfere with differentiation of naive cells into Th17 cells, resulting in a diminished supply of these cells to the pancreas.

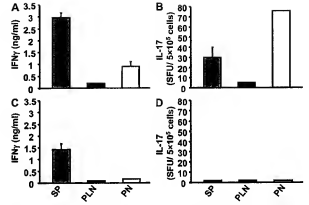

Figure 7. Splenic IFNγ induced by Ig-GAD2 treatment diminishes splenic and pancreatic IL-17 causing reversal of diabetes. IFNγ and IL-17 cytokine responses of splenic, pancreatic, and pancreatic lymph node cells from mice treated with Ig-GAD2 for 1 wk (A and B) or 25 wk (C and D) starting from the week of hyperglycemia diagnosis. The cells were stimulated with GAD2 peptide, and the responses were measured by ELISA for IFNγ and ELISPOT for IL-17, as indicated in Materials and methods. Each bar represents the mean ± SD of two independent experiments.

DISCUSSION

Treatment with anti-CD3 antibody alone has been shown to reverse diabetes, but disease reoccurrence has been observed (30–32). This justifies the search for new strategies, and the combination of anti-CD3 antibody with antigen-specific therapy did overcome rebounding of disease (33). Because T1D involves multiple autoantigens that likely manifest their activities at different stages of the disease, it has been difficult to define antigen-based regimens that could reverse the disease process at an advanced stage (1). Because Igs have proven powerful for enhancing tolerogenic function of peptides (12, 16) and GAD2 peptide was defined as a late-stage protective epitope (5–8), we incorporated GAD2 peptide onto an Ig molecule and tested the resulting Ig-GAD2 for protection, as well as reversal of advanced T1D process. Surprisingly, Ig-GAD2 was not protective at the preinsulitis stage (Fig. 1 C), but delayed the disease when it was administered upon IAA seroconversion (Fig. 1 D) (14). This differential efficacy may be related to delayed spreading of GAD2-specific T cells that become available for targeting at an advanced stage of the disease (7). This has prompted us to test for reversal of T1D at the prediabetic stage, when blood glucose has reached an abnormal level. Again, Ig-GAD2 was able to restore long-lasting normoglycemia in most of the animals (Fig. 2, A and C), and when the regimen was extended to 25 wk (Fig. 2, B and D) all mice were protected. To date, we have tested >30 mice with the 25-wk regimen and all animals maintained normoglycemia until they were terminated at 52 or 56 wk of age for other use. At the histology level, the 15-wk regimen reduced infiltration, and this was accompanied by an increase in the total number of islets relative to the beginning of the treatment (Fig. 3 B). However, with the 25-wk regimen, the number of islets dropped back to where it was at the hyperglycemic stage, but the majority of these were healthy islets. We believe that both eradication of infiltration from slightly infiltrated islets as well as formation of new islets were part of the repair process. The initial increase and the return of the number of islets may reflect dynamics in which new islets were formed while severely infiltrated islets were eliminated and those mildly infiltrated were cleaned up. This is drawn from Fig. 3 C, which illustrates the evolution of status and number of islets during the treatment and is supported by the BrdU incorporation observed with insulin-producing β cells (Fig. 4). There was, in fact, a substantial increase of dividing cells that were simultaneously producing insulin, in addition to cells that were producing insulin without evidence of division (Fig. 4). The formation of new islets has been reported before, but whether these are the product of stem cells maturation, origination from splenic cells, or division of residual β cells remains unknown (34–38). It has been shown that physical damage of islets stimulates β cell division (38). In Ig-GAD2–treated mice, upon clearance of inflammatory cells the damaged islets may likewise spontaneously initiate division of β cells. However, given that exogenous compounds such as CFA (35–37) and how Ig-GAD2 allow for β cell division, it may be that clearance of inflammatory cells minimizes cell division inhibitory factors, leading to proliferation of residual β cells or differentiation of β cell progenitors. The precise mechanism underlying β cell proliferation is of great interest, and

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effort is being made to determine how Ig-GAD2 leads to β cell division. What is important here is that a single-epitope treatment could lead to eradication of infiltration involving diverse T cell specificities. One has to assume that there must be at least a local bystander suppression that targets GAD2-specific T cells and unrelated neighboring cells. When the mice treated with Ig-GAD2 were tested for cytokine production, we expected to see suppressive or Th2-associated cytokines, which usually drive bystander suppression. The results, however, showed that there was IL-10 production, but that this was accompanied by IFNγ (Fig. 5). The other surprise was that neutralization of IFNγ, but not IL-10, nullifies the therapeutic action of Ig-GAD2 and restores diabetes (Fig. 5). These findings provide support to prior observations showing that TCR transgenic IFNγ-producing GAD2-specific T cells prevent the onset of diabetes in an animal model of disease transfer (8). The question then is how can a well-defined inflammatory cytokine such as IFNγ mediate suppression of diabetes, which likely involves diverse T cell specificities? Given the recent observations indicating that IFNγ could interfere with differentiation of naive cells into Th17 (9, 10), and that IL-17, which is the product of Th17, displays pathogenic functions (39), we sought to test whether progression to diabetes involves the activity of Th17 and if so whether treatment with Ig-GAD2 affects these pathogenic T cells. Indeed, an increase of IL-17 was observed in NOD mice as they progressed toward diabetes (Fig. 6 A), and treatment with Ig-GAD2 reduced the frequency of IL-17–producing Th17 cells (Fig. 6 B). However, neutralization of IFNγ by anti-IFNγ antibody restored IL-17 production (Fig. 6 B). In support of this Ig-GAD2–induced IFNγ/IL-17 interplay is the observation that administration of rIL-17 with Ig-GAD2 nullified the therapeutic effect of Ig-GAD2. Also, neutralization of both IFNγ and IL-17 support protection, further justifying the IFNγ/IL-17 interplay. Moreover, polarized BDC2.5 Th17 cells transferred diabetes to NOD.scid mice, and neutralization of IL-17 inhibited such disease transfer (Fig. 6 F). Finally, IFNγ is mostly produced in the spleen, which provides a noninflammatory environment (Fig. 7, A and B) and likely acts to inhibit differentiation of naive cells into Th17 in this organ, leading to a diminished supply of pathogenic Th17 cells into the pancreas. In fact, upon treatment with Ig-GAD2, Th17 cells become undetectable in the spleen or pancreas, whereas IFNγ remained significant in the spleen to sustain a long-lasting inhibition of Th17 differentiation (Fig. 7, C and D). It is known that IFNγ signaling through IFNγ receptor (IFNγR), in conjunction with other inflammatory cytokines, interferes with β cell growth and induces apoptosis (40, 41). In the Ig-GAD2 treatment, the fact that IFNγ is produced in the spleen may play dual beneficial roles. It inhibits differentiation of pathogenic Th17 cells, allowing for clearance of infiltration and termination of islet inflammation and by being away from the islets its interference with β cell growth and death is prevented, hence proliferation of β cells. This also provides support to the dual pathogenic/protective role IFNγ plays in diabetes, which likely depends on the site of production and T cell differentiation (42). In fact, this goes well with the observation that neutralization of IL-17 did not protect against diabetes transferred by IFNγ-producing Th1 BDC2.5 cells, as these lymphocytes could home to the pancreas, where their IFNγ drives apoptosis of β cells (Fig. 6, E and F).

Overall, we suggest that adjuvant-free Ig-GAD2 induced the production of IFNγ in a noninflamed lymphoid organ, leading to inhibition of differentiation of naive cells into Th17 cells, culminating in diminished infiltration, formation of β cells and reversal of the diabetic process. The presence of IFNγ would inhibit differentiation of neighboring naive cells, thus suppressing diverse T cell specificities. For effective bystander suppression to occur, it may be that Th1 cells migrate to the PLN and inhibit differentiation of diverse T cells into Th17 cells. However, because Th1 cell were not detected in this organ, the likely alternative is that APCs loaded with β-cell antigens circulate from the pancreas to the spleen and subject diverse T cells to inhibition of differentiation by local IFNγ-producing GAD2-specific Th1 cells. Administration of exogenous IFNγ may protect against diabetes if targeted to the site of T cell differentiation during antigen stimulation, but away from the islets. It is also important to mention that the regimen is effective at late stages, but not before insulitis, possibly because availability of GAD2-specific T cells and production of IFNγ are delayed. In fact, Ig-INSβ was able to delay the disease when given at the preinsulitis stage (16), but was unable to counter the disease once the mice had progressed to the hyperglycemic stage (not depicted). Again, this supports the dynamics of different epitopes during disease initiation and progression.

Collectively, the findings suggest that this antigen-specific immunomodulation targets diverse pathogenic T cells to halt inflammation and drive an islet repair process that restores long-lasting normoglycemia.

MATERIALS AND METHODS

Mice

NOD (H-2^g), NOD.BDC2.5, and NOD.scid mice were used according to the guidelines of the University of Missouri Columbia Animal Care and Use Committee.

Peptides

All peptides used in this study were purchased from Metabon and purified by HPLC to >90% purity. Glutamic acid decarboxylase 2 (GAD2) peptide corresponds to aa residues 206–220 (TYEIAPVFVLLEVVT) of GAD-65 (7). Hen egg lysozyme (HEL) peptide encompasses a nondiabetogenic epitope corresponding to aa residues 11–25 (AMKRHGLDNYRGYSL) of HEL (43). GAD2 and HEL peptides are presented to T cells in association with I-A^g MHC class II molecules.

Ig chimeras

Ig-GAD2 and Ig-HEL express GAD2 and HEL peptide, respectively. This was accomplished by inserting the corresponding nucleotide sequence in place of the diversity segment within the complementarity determining region 3 (CDR3) of the heavy chain variable region of the 91A3 IgG2b, κ Ig (13–16). The fusion heavy chain gene was then transfected along with the parental κ light chain gene for expression as a complete self-Ig molecule, as previously described (11, 13–16). Large-scale cultures of transfectoma cells were performed in DME media containing 10% iron-enriched calf

serum (BioWhittaker). Purification of the chimeras used separate columns of rat anti–mouse κ chain mAb coupled to CNBr-activated 4B Sepharose (GE Healthcare).

Islet cell purification

This was done according to a standard islet purification procedure (44). In brief, the pancreata were digested with collagenase type IV (Invitrogen), and islets were separated on a ficoll gradient (GE Healthcare).

T cell line and proliferation assay

A T cell clone specific for GAD2 peptide was generated in NOD mice as previously described (15). For presentation of Ig-GAD2, irradiated (3,000 rads) NOD female splenocytes (5×10^5 cells/50 μl/well) were incubated with graded amounts of either free peptide or Ig chimeras (100 μl/well), and 1 h later the GAD2-specific T cells (5×10^4 cells/well/50 μl) were added. Proliferation was measured by $[^3H]$thymidine incorporation assay.

Assessment of insulin autoantibody (IAA) seroconversion, hyperglycemia, and diabetes

Serum IAA was detected by ELISA using porcine insulin as antigen, as previously described (16). Assessment of blood glucose levels used test strips and an Accu-Chek Advantage monitoring system. A mouse is considered hyperglycemic or diabetic when the blood glucose level is 160–250 mg/dl or 300 mg/dl, respectively, for 2 consecutive weeks.

Ig-GAD2 treatment regimens

Treatment at the preinsulitis stage. Mice are given an i.p. injection of 300 μg Ig-GAD2 or Ig-HEL in 300 μl PBS at 4, 5, and 6 wk of age, a stage at which islet infiltration has begun and that is referred to as preinsulitis. The mice were monitored for blood glucose level up to 30 wk of age.

Treatment at the insulitis (IAA^+) stage. Mice are tested for IAA, and those who seroconvert between the ages of 8–11 wk are given a weekly i.p. injection of 300 μg of Ig-GAD2 or Ig-HEL in 300 μl PBS up to week 12. Subsequently, the mice received another 300 μg of Ig-chimera every 2 wk until the age of 24 wk. These mice were monitored for blood glucose level beginning at week 12 until 30 wk of age.

Treatment at the hyperglycemic stage. Mice began blood glucose level monitoring at 12 wk of age, and those who displayed a level of 160–250 mg/dl for 2 consecutive weeks between the ages of 14–30 wk were considered hyperglycemic. These mice were then subjected to a daily i.p. injection of 500 μg Ig-GAD2 or Ig-HEL for 5 d. Subsequently, the mice received another 500 μg of Ig-chimera every week for 15 or 25 consecutive weeks, and blood glucose levels were continuously monitored until 56 wk. These treatments are referred to as 15- and 25 wk-treatment regimen, respectively.

Histology

Pancreata were harvested from NOD females, fixed in 10% formalin, and embedded in paraffin. Sections of 8-μm thickness were cut 100 μm apart to prevent double counting the same islet. Four sections per pancreas were stained with hematoxylin and eosin and analyzed by light microscopy. Insulitis scoring was performed according to the following criteria: severe insulitis, 50% or higher of the islet area is infiltrated; mild insulitis, <50% of the islet area is infiltrated; periinsulitis, infiltration is restricted to the periphery of islets; and no insulitis, absence of cell infiltration.

Immunohistochemistry

Evaluation of cell division by insulin-producing β cells was done as follows: Ig chimera–treated mice were injected i.p. with 100 mg/kg of BrdU in PBS (Sigma-Aldrich), 3 h before euthanasia. Pancreata and intestine were harvested and fixed, and sections were prepared as described in the previous section. For assessment of insulin production, the sections were stained with primary guinea pig anti-insulin antibodies, incubated with biotinylated goat anti–guinea pig antibodies, and visualized by saturation with Streptavidin-alkaline phosphatase (using the chromagen, 5-Bromo-4-chloro-3-indolyl phosphate/Nitroblue tetrazolium. For detection of BrdU incorporation, the sections were counter stained with biotinylated anti-BrdU antibody (Zymed), treated with Streptavidin–horseradish peroxidase, and visualized with the chromagen 3-amino-9-ethylcarbazole.

Cytokine assays

Splenocytes (5×10^5 cells/well) were incubated with 30 μg/ml of free peptide or 5 μg/ml anti-CD3 antibody for 48 h, and cytokines in the supernatant were measured by ELISA and ELISPOT, as previously described (45).

Flow cytometry

For staining of CD4, CD25, and CD62L, cells were harvested from spleens and pancreatic lymph nodes and incubated with anti-CD4-PE, biotin-conjugated anti-CD25 (or isotype control biotin-conjugated rIgM), and anti-CD62L-FITC (or isotype control rIgG2a-FITC) for 30 min at 4°C. Subsequently, the cells were washed and stained with PerCP-conjugated streptavidin for 30 min at 4°C. The cells were washed, fixed with 4% formaldehyde for 20 min at room temperature, and then analyzed. All antibodies were purchased from BD PharMingen.

For intracellular Foxp3 staining, cells from spleens and pancreatic lymph nodes were first stained with anti-CD4-PE and biotin-conjugated anti-CD25 antibodies. This was followed by PerCP-conjugated streptavidin staining. The cells were fixed with Fix/Perm buffer (eBioscience), washed with permeabilization buffer (eBioscience), and stained with anti-Foxp3-FITC antibody (clone FJK-16s; eBioscience), or isotype control rIgG2a-FITC.

For intracellular cytokine analysis of IL-10 and IFNγ, the splenic cells (2×10^6 cell/ml) were stimulated with free peptide (30 μg/ml) for 6 h followed by 10 h incubation with brefeldin A (10 μg/ml) to block cytokine secretion and facilitate intracellular accumulation. The antibodies used were PerCP-anti-CD4 (RM4-5), biotin-anti-Vβ8.1/8.2, PE-anti-IFNγ (XMG1.2), and FITC-anti-IL-10 (JES5-16E3; all from BD Biosciences). Isotype-matched controls were included in all experiments. Events were collected on a FACScan flow cytometer and analyzed with CellQuest software (Becton Dickinson).

T cell polarization

Naive splenocytes were isolated from 4–wk-old NOD.BDC2.5 transgenic mice and activated with soluble anti-CD3 (5 μg/ml) and anti-CD28 (5 μg/ml) antibodies for 3 d in 10% FCS–DME media under Th17 polarizing (TGFβ [3 ng/ml], IL-6 [20 ng/ml], anti-IFNγ antibody [10 μg/ml], and anti-IL-4 antibody [10 μg/ml]) and nonpolarizing conditions. Supernatant from activated cells was tested for IFNγ and IL-17 by ELISA, and the cells were used for adoptive transfers.

Adoptive transfer experiments

For disease transfer by Th17, 10×10^6 naive, nonpolarized and Th17-polarized cells were injected i.v into NOD.scid (4–6–wk-old) mice. Additional groups of mice received IL-17–neutralizing antibody along with the T cell transfer to serve as controls. Anti-IL-17 antibody (TC11-18H10; 200 μg/mouse) was given on the day of transfer, and 2 additional injections were given at day 4 and 16 after transfer.

Statistical analysis

The χ^2 test was used for incidence of diabetes analysis among experimental and control groups. For the rest of the experiments, P values were calculated with the two-tailed Student's unpaired *t* test.

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